METHOD FOR HALOGENATED FURANONES (MX-ANALOGUES)

METHOD SUMMARY

For the Nationwide DBP Occurrence Study, a method was developed for the analysis of the following halogenated furanones: MX, MCA, BMX-1, BMX-2, and their *open* forms (see full names in Glossary; structures in Figure 1). This method evolved from the previous methods of Holmbom et al. (1981), Hemming et al. (1986), and Kronberg et al. (1988, 1991) which required large volumes of water for concentration onto XAD resins and lengthy processing times that endanger the stability of the MX-analogues. Because of their complexity, these methods do not incorporate adequate quality assurance (QA)/quality control (QC) components to validate their resulting data. In order to accurately assess the concentrations of MX-analogues in drinking water, a liquid-liquid extraction (LLE)-gas chromatography (GC)-electron capture detection (ECD) method was developed, which uses smaller sample volumes and shorter processing times to protect compound stability.

For the new method, the chlorine quenching agent, ammonium sulfate [100 μL of 40 mg/mL $(NH_4)_2SO_4$] was added to acid-washed amber glass sample bottles (250 mL) fitted with Teflon-lined screw caps prior to sending the bottles to the water treatment plants for duplicate sample collection. Field blanks filled with DIW were included. Sample bottles were returned to UNC in a cooler with ice packs, shipped by overnight delivery. Immediately upon arrival, or within 5 hours, the samples were removed from the cooler, and analyzed for MX and MCA after they had reached room temperature (the BMX analysis was performed one week following receipt of samples). The calibration samples were prepared on the day of extraction, at 0, 50, and 250 ng/L MX and MCA (or 0, 100, and 500 ng/L BMX-1,2,3) in DIW in 250 mL volumetric flasks. One sample from each plant was collected in a 1 L amber bottle to allow for a matrix spike sample (250 ng/L MX and MCA or 500 ng/L BMX-1,2,3).

Prior to extraction, each 250-mL sample was spiked with MBA as a surrogate standard at 250 ng/L, and acidified to pH 2 with sulfuric acid. Each sample was extracted twice with 50 mL of MtBE in a 500 mL glass separatory funnel. The combined extract was collected in a 125 mL amber bottle (fitted with a Teflon-lined screw cap) containing two approximately 8 g of calcium chloride ($CaCl_2$), and shaken to remove residual water dissolved in the MtBE. The extract was transferred (without $CaCl_2$) to a 250 mL round bottomed flask and reduced to a few mLs by rotary evaporation at 40°C. The reduced extract was transferred to a 20 mL centrifuge tube, with a few mL rinse of MtBE. This extract was further reduced to about 500 μL by nitrogen (N_2) gas. To this reduced MtBE extract was added 2 mL of 14% BF_3/MeOH, and the tube was sealed with a Teflon-lined screw cap. The solution was mixed and heated at 70°C for 4 hours in an oven. After returning to room temperature, the derivatization agent and pH were neutralized by adding 4 mL of 10% $NaHCO_3$, with mixing.

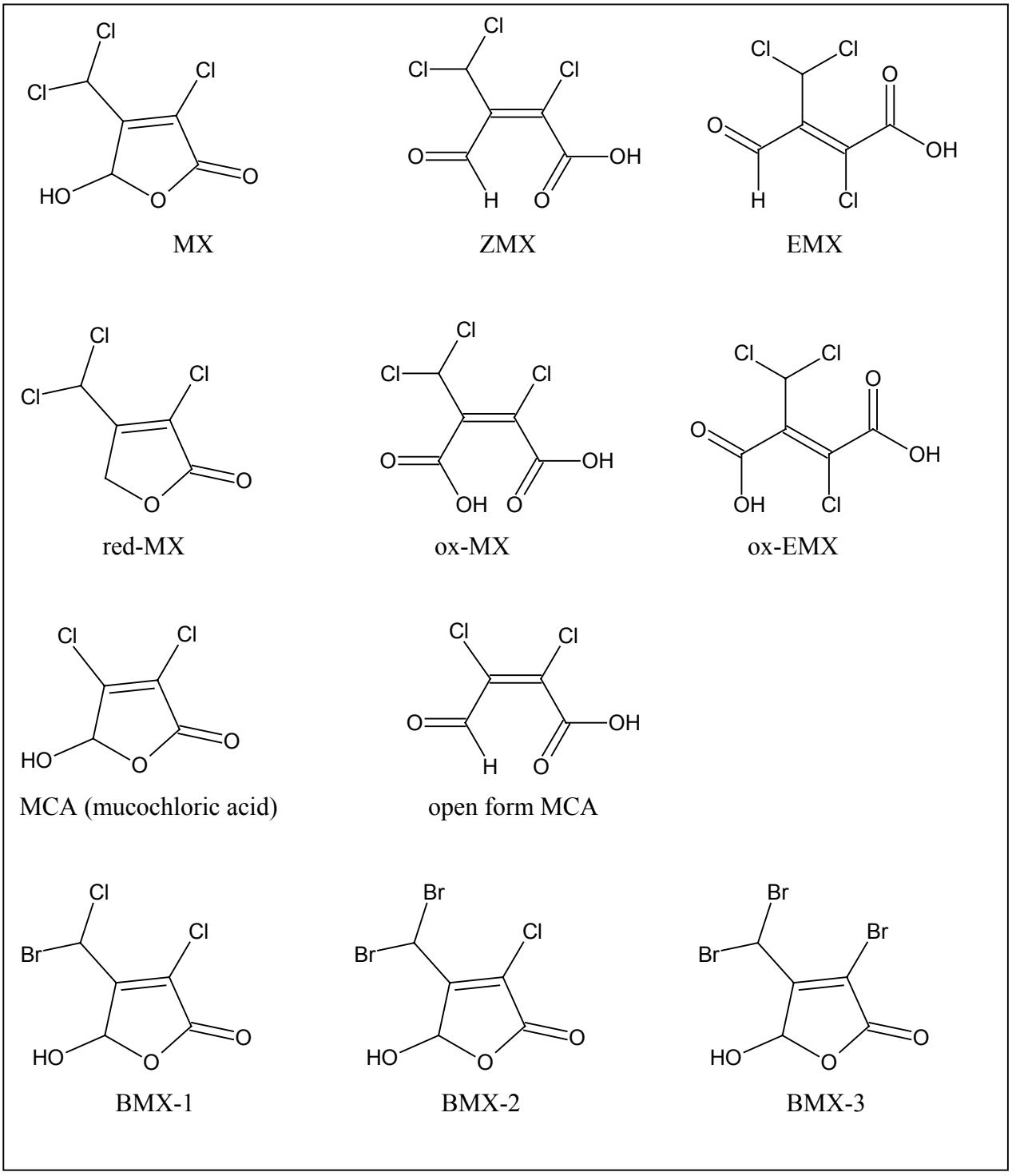

Figure 1. Structures of halogenated furanones (MX-analogues).

The MXR-analogues were back-extracted twice into 1 mL hexane. The combined 2 mL hexane extract was collected in a 10 mL centrifuge tube and reduced to <250 μL by N_2 gas. The internal standard hexachlorobenzene (HCB) was added (5 μL of 500 ng/mL HCB/hexane) to the hexane extract, which was brought to a final volume of 250 μL. The final hexane extract was transferred to an amber crimp-topped vial with a 300 μL glass insert for GC-ECD analysis. The MX and MCA samples were separated by gas chromatography on a HP-5MS column (30-m x 0.25 mm ID x 0.25 μm film thickness) at a temperature program of 105°C for 1 min, 2.5°C/min to 140°C, and 20°C/min to 280°C, with an injection temperature of 200°C and a detector temperature of 300°C. The BMX samples were separated by gas chromatography on a Phenomenex ZB5 column (60-m x 0.25 mm ID x 0.25 μm film thickness) at a temperature program of 100°C for 1 min, 20°C/min to 150°C, 1°C/min to 185°C, and 20°C/min to 280°C, with an injection temperature of 160°C and a detector temperature of 300°C. Calibration curves for each component were constructed using analyte area relative to the internal standard (HCB). Calculated concentrations of analytes were corrected by percent recovery in the matrix spike sample. Relative areas of the analytes to the surrogate standard (MBA) were not reliable for duplicate calibration samples.

Because method development continued during the first year of plant surveys, no halogenated furanone data is presented during the first two seasons. The plant data and discussion is included among the results for each utility elsewhere in this report. The minimum reportable limit for MX-analogues was 40 ng/L. Non-zero concentrations below 40 ng/L are given in parentheses, to indicate relative values extrapolated from the calibration curves.

INTRODUCTION

The detection of the disinfection by-product (DBP) 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) in chlorinated drinking water in Finland in the early 1980's caused great concern in the scientific and public health communities because MX was found to account for 20-60% of the mutagenicity in chlorinated drinking water. Later research showed that MX was also carcinogenic to rats (at a dose of 400 μg MX per kg body mass per day) (Komulainen et al., 1997). Other compounds similar to MX (referred to as MX-analogues), including ZMX, EMX, red-MX, ox-MX, mucochloric acid (MCA), and brominated forms of MX--BMX-1, BMX-2, BMX-3 (Figure 1) have also been identified in drinking water.

Following the initial identification of MX in Finland (Kronberg and Vartiainen, 1988), MX and MX-analogues were also detected in drinking waters from the United States, the United Kingdom, Australia, Canada, Spain, China and Japan, in levels ranging from 0.1 to 90 ng/L (Andrews et al., 1990; Horth, 1990; Huixian et al., 1995; Meier et al., 1987; Simpson and Hayes, 1993; Simpson and Hayes, 1998; Smeds et al., 1995; Suzuki and Nakanishi, 1990; Wright et al., 2002). MX has been detected primarily in waters treated with chlorine, less so with the use of chlorine dioxide or chloramines, and very minimally in ozonated waters with post-chlorination (Holmbom and Kronberg, 1988).

The structural components responsible for the mutagenicity of MX are the $CHCl_2$ and Cl substituents in a *cis* arrangement on a carbon-carbon double bond (Figure 1). The mutagenity of these substituents is enhanced by incorporation into the 5-hydroxy-2(5H)-furanone *ring* system or an *open* structure that can readily transform to this ring system under the conditions of mutagenic testing (Ishiguro et al., 1987). Therefore, when comparing the relative mutagenicities of the MX-analogues (Figure 1), EMX, ox-EMX and MCA are less mutagenic than the other MX-analogues. The mutagenicity of halogenated furanones is also enhanced by the presence of the C-5 hydroxyl group (Kronberg and Franzen, 1993), making red-MX less mutagenic than MX (LaLonde et al., 1991). Bromine substitution with chlorine substituents can increase the toxicity of the compound, as found for THMs and the BMX-analogues (Bull, 1993; Ramos et al., 2000). The bromine substituents originate from natural bromide ions found in many coastal ground and surface waters.

While mutagenicity in *Salmonella* cannot be used to determine carcinogenicity in humans, MX is still considered a potential human carcinogen. Because MX and other analogues are highly mutagenic and there is very little occurrence data for them (particularly for the brominated-MX analogues), they received a high priority for inclusion in this Nationwide DBP Occurrence Study

ANALYTICAL METHOD DEVELOPMENT

Previous Methods

Method development for the detection of MX in drinking water began in the 1980's, at first catalyzed by Holmbom's identification of MX in kraft chlorination effluent (Holmbom et al., 1981). Soon after, Hemming et al. (1986) and Kronberg et al. (1988) detected MX in chlorinated drinking waters. The methods of Hemming et al. (1986) and Kronberg et al. (1988) became the key methods that were used to detect MX thereafter.

The stability of MX is very sensitive to the pH of an aqueous solution. The *ring* form is predominant at low pH, but as the pH rises, the ring opens to ZMX, which tautomerizes to EMX, and at higher pH levels (above pH 8), degrades to smaller products (Kronberg and Christman, 1989, Figure 2). Hemming et al. (1986) and Kronberg et al. (1988) adjusted the pH to stabilize the *ring* form. The extraction method consisted of acidification of a large volume sample (10 L), concentration on a mixture of XAD resins, elution with ethyl acetate, and solvent reduction to dryness by rotary evaporation and nitrogen gas. Methylation of the hydroxyl group on the MX ring structure was achieved by heating with sulfuric acid in methanol (Figure 3).

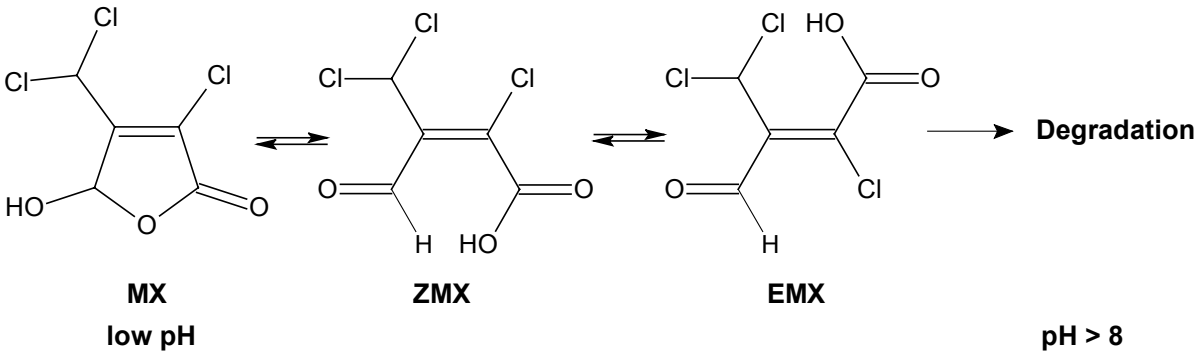

Figure 2. MX degrades as pH increases.

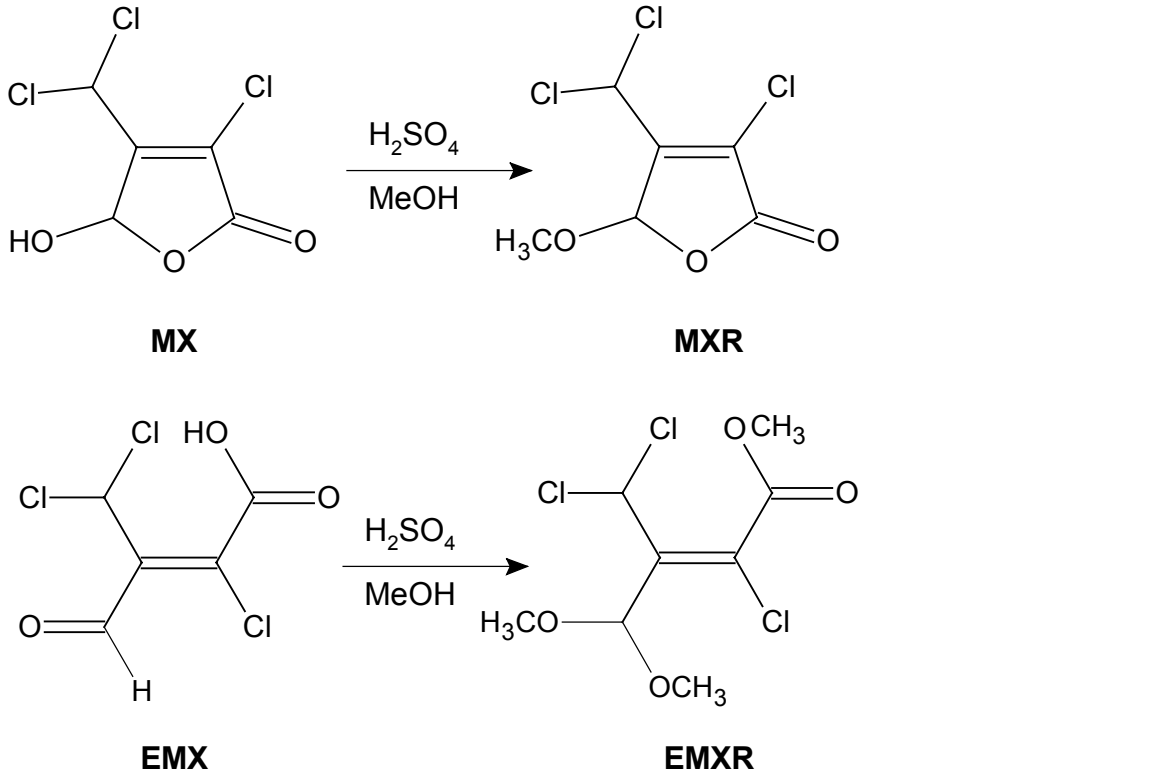

Figure 3. Methylation of MX-analogues with sulfuric acid in methanol.

Methylation converts the alcohol group on the MX ring to a methyl ether group, but the carboxylic acid groups of the *open* forms of MX (ZMX and EMX) are changed to esters, and the aldehyde groups to dimethyl acetal groups (Figure 3). Thus, to simplify naming the methylation products, they are all referred to as "esters," i.e. MX becomes MXR. The esterified MX (MXR) was recovered by neutralization with sodium bicarbonate aqueous solution, and back-extraction into hexane. The reduced hexane extract (100 μL) was analyzed by capillary gas chromatographic (GC) separation and high resolution mass spectrometric detection (HRMS), with a detection limit of 2 ng/L MX. In some cases, researchers used high performance liquid chromatography (HPLC) prior to methylation to remove natural organic carbon contaminants (Kronberg et al., 1985a; Meier et al., 1987). The HPLC separation involved first concentrating XAD extracts of drinking water to dryness by rotary evaporation, followed by soxhlet extraction with diethyl ether (Et_2O), extraction with 2% sodium bicarbonate to remove strong acids, acidification of the aqueous phase to pH 2 with HCl, re-extraction with Et_2O, transfer to 30% methanol/water, separation into 2 mL fractions by a C18 semi-prep column using a 30-100% methanol/water gradient, followed by a 100% hold for 10 min, methylation of the weak acid fractions, and detection of MXR-analogues by GC/MS (Meier et al., 1987). Other researchers applied a silica column clean-up step to the final hexane extract (Suzuki and Nakanishi, 1995), or multiple reaction monitoring during mass spectrometric detection (Simpson and Hayes, 1993) to isolate the MX-analogues from interfering co-contaminants such as natural organic matter (NOM).

Identification of MX-analogues. The structure of MX was first determined by HRMS, UV and IR spectroscopy (Holmbom et al., 1981). Padmapriya et al. (1985) reported the IR, UV, and ^{1}H NMR spectra for MX and MXR, and the ^{13}C NMR spectrum for MX. No identification spectra have been previously published for ZMX. Kronberg et al. (1988) identified EMX by its ^{1}H NMR and mass spectra, and EMXR by its mass spectrum. Kronberg et al. (1991) identified ox-EMX, ox-EMXR, ox-MXR and red-MX by their mass spectra. LaLonde et al. (1990) identified red-MX by its IR, ^{1}H NMR, and ^{13}C NMR spectra, and MCA by its ^{1}H NMR spectrum. Nawrocki et al. (2000) identified MCR by its mass spectrum. Lloveras et al. (2000) identified BMX-1, BMX-2, and BMX-3 by their ^{1}H NMR, ^{13}C NMR and mass spectra. Peters (1991) identified BMXR-1, BMXR-2, and BMXR-3 by their mass spectra.

Derivatization Efficiency. Kronberg et al. (1988) achieve derivatization of MX by addition of 2% sulfuric acid in methanol (H_2SO_4/MeOH, Figure 3), heated at 70°C for 1 hour. While the efficiency of this reaction has not been reported for the derivatization of MX, some researchers have compared the use of H_2SO_4/MeOH to other derivatization agents. Diazomethane (CH_2N_2) does not successfully methylate MX and its analogues (Kronberg et al., 1991). Although H_2SO_4/MeOH can adequately methylate MX, it cannot methylate the diacidic MX-analogues (ox-MX and ox-EMX). A 14% boron trifluoride methanol complex (BF_3/MeOH) solution, heated at 70°C for 12 hours, was successfully applied to ox-MX and ox-EMX (Kanniganti et al., 1992). Meier et al. (1987) claimed that the derivatization yield of EMX is related to the derivatization time (using Amberlite IR 120 sulfonated polystyrene cation exchange resin in methanol, in a sealed tube, at 70°C for 16-18 hours). Huixian et al. (1995) compared the MXR yield from derivatization with

saturated BF_3/MeOH to the method with 2% H_2SO_4/MeOH, and found that saturated BF_3/MeOH was the more efficient derivatization agent regardless of reaction time (1-8 hours at 95°C in water bath). Overall, BF_3/MeOH has shown to be the best derivatization agent, with reaction time significantly affecting the product yield.

Extraction Efficiency. Holmbom et al. (1984) evaluated a number of organic solvents and solid phases to extract MX from aqueous solutions; mutagenicity was measured as an indicator of MX recovery. Ethyl acetate (EtAc) completely extracted the mutagenicity (70-90%), while dichloromethane (50-70%) and pentane (<10%) recovered less of the mutagenicity. Rotary evaporation of EtAc extracts did not degrade the mutagenicity (even after 10 min at 40°C and 1.5 kPa). Adsorption of MX onto XAD-4 resin recovered similar amounts of mutagenicity as EtAc. Although MX can ionize in aqueous solution, anion-exchange solid phase materials are not appropriate for isolating MX from chlorinated aqueous samples. MX behaved as a neutral compound when applied to the anion exchange DEAE-Sepharose column due to the MX ring structure.

Acidification prior to resin adsorption (XAD-2/8 resin adsorption/acetone elution) was essential for adequate recovery of MX in the protonated form (Figure 2) from spiked water samples and to maintain the stability of MX at low pH (Meier et al., 1987). MX was measured in terms of mutagenicity assays. XAD-2/8 recovery of mutagenicity from acidified (pH 2), chlorinated MX-spiked drinking water samples was only 55% effective. Subsequent extraction and HPLC isolation recovered only 18% of the remaining MX, resulting in an overall 10% MX recovery through XAD-2/8 adsorption, Et_2O extraction, HPLC separation, and derivatization procedures. These percent recoveries were not taken into account when reporting MX concentrations, and no apparent method calibration solutions were analyzed to monitor recoveries at different MX concentrations. MX concentrations were determined relative to a derivatized MX standard by high resolution GC/MS analysis. Recoveries of MX from water samples buffered at higher pH levels (pH 8) were 0-1%; the high pH favors MX in the ionized form and does not promote extraction from aqueous solution. Poor extraction recovery of MX from drinking water onto XAD resins was also attributed to complexation with chlorinated humic materials. When evaluated separately, the methyl-methacrylate polymer XAD-8 recovered more MX than the styrene-divinyl benzene copolymer XAD-2 (92 vs. 22 % MX recovery) from a fortified deionized water sample (20 L, 50 ng/L MX) at pH 2; MX recovery was measured by mutagenicity (Schenck et al., 1990). MX recovery was also significantly enhanced by reducing XAD-8 adsorption time; a total sample collection time of 25 hours recovered 92 % MX, whereas 56 hours recovered only 38 % MX (see stability section).

The octanol-water partition coefficient, K_{ow}, is indicative of how much of an analyte is likely to partition out of water into a highly polar organic solvent. MX is fairly hydrophilic with a K_{ow} of 11.9 (mg/L octanol / mg/L water) at pH 2 (Holmbom et al., 1984). The K_{ow} value should be lower in neutral pH surface and drinking waters, and therefore MX is less susceptible to bioaccumulation in these waters. The K_{ow} of MX *open* (ZMX or EMX) in the neutral acid form was computed to be 1.16, using CLOGP, ver3.5 (Biobyte Inc., Pomona, CA) (DeMarini et al., 2000). The variability of these K_{ow} values is likely due to the difference between the *ring* and *open* forms of MX, and the pH considered.

Kronberg et al. (1991) used mucobromic acid (MBA, Figure 4) as an internal standard to assess recovery of MX-analogues through the derivatization process, by spiking MBA into the EtAc extract prior to derivatization (derivatization standard). However, MBA was determined to be an inappropriate surrogate standard (by spiking MBA into the original water sample prior to acidification and XAD adsorption) for the XAD/HPLC MX method (Simpson and Hayes, 1993), because MBA is more susceptible than MX to intermolecular hydrogen bonding with natural organics. The levels of MX recorded were corrected for recovery losses, based on separate MX method recovery experiments (average 10% recovery, consistent with Meier et al. 1987). Higher levels of total organic carbon (TOC) in drinking water have been associated with lower recovery of MX (Meier et al. 1987). The high K_{ow} (11.9 mg/mg) for MX, may indicate the likelihood that MX would strongly associate with NOM as a highly polar solvent, and not be easily extracted by XAD. The major loss of MX was seen in the HPLC fractionation steps (average 60% recovery in this step, Simpson and Hayes, 1993), but these steps are only necessary in high TOC waters. Multiple reaction monitoring (MRM) by mass spectrometry was investigated as an alternative method to HPLC for removal background natural organic interferences, and it showed some promise (Simpson and Hayes, 1993). MRM eliminates interference from co-extracting compounds by monitoring compound-specific metastable transitions between selected parent and daughter ions of the target analyte.

MBA *ring* **MBA *open***

Figure 4. Mucobromic acid (MBA) isomers.

Stability of MX-Analogues. MX hydrolysis, isomerization, and decomposition processes in aqueous solution are strongly dependent on pH (Holmbom et al., 1989). MX is stable at pH 2 but starts to degrade at pH 4 and above. Beyond pH 6.5, the water solubility of MX increases rapidly, due to ring opening and dissociation (tautomerization), as determined by extraction of aqueous MX solutions with ethyl acetate at different pH values (Holmbom et al., 1984). The degradation of MX at pH 5-7 correlates with the formation of EMX (Simpson and Hayes, 1993). However, EMX also degrades over time at neutral or alkaline pH (Holmbom and Kronberg, 1988). When acidified to pH 2, EMX completely converts to MX. The BMX-analogues also show tautomerization, degrading over time (48 hours) from the *ring* forms to the *open* forms and finally to degradation products, as measured in a pH 7.4 phosphate-buffered aqueous solution by HPLC/UV (Ramos et al., 2000), similar to MX in Figure 2.

Meier et al. (1987) measured the mutagenic activity of MX spiked distilled water samples at 4°C. It was constant at pH 2, 4, and 8 over 14 days, but declined to 30% at pH 6 after 14 days. At 23°C, the order of stability was pH 2 > pH 4 > pH 8 > pH 6, where pH 2 was constant. The loss of activity in pH 4-8 followed first-order decay kinetics. ZMX occurred in MX solutions buffered at pH 6, but less at pH 8 (stored for 7 days at 23°C). The pK_a value of MX was determined to be 5.3 by NMR spectroscopy (Streicher, 1987). However, the pK_a of MX *open* (ZMX or EMX) was computed to be 1.85, using the SPARC method (DeMarini et al., 2000). The variability of these two pK_a values is likely due to the difference between the *ring* and *open* forms of MX.

Meier et al (1987) determined the half-lives of MX in distilled water at 23°C to be 12.9 days at pH 4, 4.6 days at pH 8, and 2.3 days at pH 6, by measuring loss in mutagenicity. When MX was spiked into tap water samples buffered at pH 6 and 8, stored at 23°C, the same losses in mutagenicity were seen as those in distilled water. This work was confirmed by measuring MX concentration at pH 2-9 in MX spiked Milli-Q water by HPLC/UV analysis (Simpson and Hayes, 1993). Simpson and Hayes (1993) recovered 95% of the original MX in pH 2 Milli-Q water stored at 20°C after 14 days. At the same temperature, the half-life of MX at pH 8 (11.3 days) was much longer than that for pH 6 (5.4 days). However, at 23°C, the half-life of MX at pH 8 was 4.6 days. This agrees with rates of hydrolysis at pH 7.0 measured by Croué and Reckhow (1989) at 20ºC, $k = 0.9 \pm 0.5 \times 10^{-6}$ s^{-1} (~0.07 $days^{-1}$) and $t_{1/2} \sim 8.9$ days.

MX has been shown to degrade in the presence of increasing concentrations of chlorine (10-100 mg/L Cl_2), buffered at pH 8 (Schenck et al., 1990; Simpson and Hayes, 1993). The second order rate constant for MX degradation by chlorine was estimated to be 32.3 L mol^{-1} min^{-1}, based on the reaction rate over the first 10 min and initial concentrations of 20 mg/L MX and 40-120 mg/L Cl_2 (Schenck et al., 1990). MX degradation was also observed at lower residual chlorine concentrations (0.5-3 mg/L Cl_2) that might be practical levels found in drinking water treatment plant effluents. Chlorine and MX reacted at about a 5:1 molar ratio, and the reaction was complete within 1 day (Schenck et al., 1990). MX can be converted to EMX, ox-MX and ox-EMX in the presence of chlorine (Simpson and Hayes, 1993). However, in the presence of chloramine (10-100 mg/L NH_2Cl), MX converts to only EMX, due to the fact that chloramine is not as strong of an oxidizing agent as chlorine. EMX, ox-MX and ox-EMX were qualitatively identified as disinfection by-products, but their levels were not quantified in these studies.

Due to the MX degradation by chlorine, some researchers tried to quench the residual chlorine prior to MX analysis. Simpson and Hayes (1993) identified *L*-ascorbic acid (Figure 5, note similar furanone structure to MX) as the best quenching agent for MX, because nucleophiles in other quenching agents (e.g., sodium thiosulfate or sodium sulfite) destroy MX by removing chlorine atoms (Croué and Reckhow, 1989). The rates of decomposition of MX significantly increase in the presence of sulfite (100 μM) at 20ºC, $k = 22 \pm 3 \times 10^{-6}$ s^{-1} and $t_{1/2} \sim 8.7$ hours (Croué and Reckhow, 1989). Suzuki and Nakanishi (1990) suggest that quenching residual chlorine is unnecessary; after acidification, their samples were purged with nitrogen gas and the residual chlorine was reduced to 0.2 mg/L;

no difference in MX concentration was observed between purged and non-purged samples. However, considering the MX degradation by chlorine observed by Schenck et al. (1990), quenching of residual chlorine is necessary for a 0.3 mg/L chlorine residual and above.

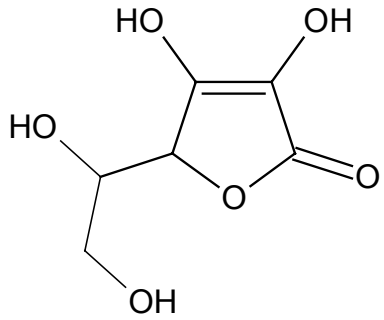

Figure 5. Structure of ascorbic acid (Vitamin C).

Summary of Current Methods for Analysis of MX-Analogues in Drinking Water

MX, ZMX, EMX, and MCA. The method of Kronberg et al. (1991) for extraction of MX, ZMX, EMX, and MCA from aqueous solutions involves first acidifying the solution to pH 2, passing the solution through a mixture of XAD-4 and XAD-8 resins (1:1), and eluting the adsorbed compounds with ethyl acetate (EtAc). However, liquid-liquid extraction has met with some success. By extracting 250 mL of a solution with successive 40, 20, and 20 mL volumes of diethyl ether, 77% of MX was recovered (Kanniganti et al., 1992). MBA was added to the EtAc extract as the derivatization standard. The EtAc extract was blown down to dryness, derivatized with 250 µL of 2% H_2SO_4/MeOH at 70ºC for 1 hour, neutralized with 2% $NaHCO_3$/deionized water (DIW), and extracted twice with 250 µL of hexane. The hexane extract was then concentrated down to 100 µL and decafluorobiphenyl was added as an internal standard. The extract was analyzed by gas chromatography on a DB-1 column (30m), with a temperature program of 110ºC for 3 min, 6ºC/min to 165ºC, and the resolved compounds detected by HRMS, single ion monitoring mode (Kronberg et al., 1991). The extract can also be separated on a DB-5 column (30-m x 0.25 mm ID x 0.25 µm film thickness), using the temperature program 50ºC for 1 min, 2.5ºC/min to 150ºC, 5ºC/min to 300ºC (Kanniganti et al., 1992).

red-MX. The method of Kronberg et al. (1991) for extraction of red-MX from aqueous solutions involves first acidifying the solution to pH 2, passing the solution through a mixture of XAD-4 and XAD-8 resins (1:1), and eluting the adsorbed compounds with ethyl acetate. Since the EtAc extract did not require derivatization, 2,3-dibromo-2(5H)-furanone (red-MBA) was added as an internal standard, and the extract was reduced to 100 µL with nitrogen gas. The EtAc extract was separated by gas chromatography on a DB-1 column (30 m), with a temperature program of 110ºC for 3 min, 6ºC/min to 165ºC. Red-MX is detected by HRMS based on retention time and most abundant ions: m/z 165 and 167 for $(M\text{-}Cl)^+$, 171 and 173 for $(M\text{-}CHO)^+$.

ox-MX and ox-EMX. The method of Kronberg et al. (1991) for extraction of ox-MX and ox-EMX from aqueous solution involves first acidifying the solution to pH 2, passing the solution through a mixture of XAD-4 and XAD-8 resins (1:1), and eluting the

adsorbed compounds with ethyl acetate. MBA was added to the EtAc extract as the derivatization standard. The EtAc extract was blown down to dryness, derivatized with 250 μL of 12% BF_3/MeOH at 100ºC for 12 hours, neutralized with 2% $NaHCO_3$/DIW, and extracted twice with 250 μL of hexane. The hexane extract was then concentrated down to 100 μL and decafluorobiphenyl added as an internal standard. The extract was analyzed by gas chromatography on a DB-5 column (60 m), with a temperature program of 160ºC for 3 min, 6ºC/min to 190ºC. Ox-EMX elutes immediately prior to ox-MX using GC/MS (HP5890 GC/VG 70-250 SEQ mass spectrometer, resolving power 1000). The LLE method using diethyl ether has also been applied successfully to these compounds (Kanniganti et al., 1992).

BMX-Analogues. The method for analysis of BMX-1, BMX-2, and BMX-3 is very similar to that of MX (Suzuki and Nakanishi, 1995). The BMX-analogues were measured in Japanese drinking waters by acidifying 10 L samples to pH 2, passing them through 50 mL XAD-8 resins, eluting with 150 mL EtAc, and concentrating down to 5 mL by rotary evaporation at 40°C. Three mL of this extract was spiked with 100 ng MBA as the derivatization standard, and evaporated to dryness with nitrogen (N_2) gas. The residue was methylated with 250 μL of 2% H_2SO_4/MeOH for 1 hour at 70°C, neutralized by 500 μL of 2% $NaHCO_3$/DIW, and extracted twice with 500 μL hexane. The hexane extract was then passed through a 500 mg Sep-Pak silica column, eluted with 1 mL hexane and 5 mL ethyl acetate:hexane (1:7), and only the last 4 mL fraction was collected and concentrated to 100 μL with N_2. Separation was achieved using a 30-m x 0.25 mm ID DB-5MS GC column, injection temperature 160°C, temperature program 50°C for 2 min, 50-120°C at 40°C/min, 120°C for 2 min, 120-135°C at 2°C/min, 135-180°C at 6°C/min, 180°C for 5 min. The components were detected by HRMS using a VG Autospec-Ultima mass spectrometer. Spike recoveries ranged from 71 to 122%.

The BMX compounds are susceptible to thermal degradation in the injection port of a GC. An injection temperature of 160°C produced a larger BMX-3 signal (HRMS) than 200°C, in a calibration range of 0-1000 pg/μL. Calibration solutions were made from standards of the esterified BMX compounds. Detection limits were also dependent on compound stability in the GC injection port. The detection limit for MX was 0.1 ng/L, whereas BMX-3 was 0.5 ng/L, using a 60,000:1 concentration factor. BMX-1 and BMX-2 showed intermediate thermal degradation (and intermediate detection limits) to MX and BMX-3.

Opportunities for Improvement of Existing Methods. A unified method needs to be developed for the analysis of all MX-analogues in drinking water in a single extract, which accounts for sample preservation and recovery of MX-analogues through each processing step. Routine analysis by GC-ECD instead of high resolution GC/MS would make the method more amenable for environmental and water treatment laboratories in the United States. Evaluation of quenching agents for residual chlorine and biocides to prevent microbial regrowth would improve sample preservation and prevent degradation of MX-analogues. Evaluating percent recoveries from each processing step based on detection of individual halogenated furanones, rather than by mutagenicity, would also prove more

valuable in the development of an analytical method for the detection of MX-analogues in drinking water.

New Method Development

Identification and Quantification of Standards. Development of a method for the analysis of MX-analogues (Figure 1) in drinking water began by first identifying and quantifying the compounds in synthesized and commercially available standards. The only commercially available MX-analogues were MX, mucochloric acid (MCA), and mucobromic acid (MBA, surrogate standard), from Sigma-Aldrich (St. Louis, MO). The other components were provided in small mg quantities from the labs of individual researchers. Leif Kronberg (Åbo Akademi, Finland) synthesized EMX (75% purity) and ox-EMX (Kronberg et al., 1991). Ramiah Sangaiah (UNC) synthesized MX, red-MX, and ox-MX (Kronberg et al., 1991; Padmapriya et al., 1985). Angel Messeguer (CSIC, Spain) synthesized BMX-1, BMX-2, and BMX-3 (Lloveras et al., 2000). Starting with MX (Sigma-Aldrich), the identities and purities of the compounds were confirmed by ^{1}H and ^{13}C nuclear magnetic resonance, electron ionization and chemical ionization mass spectrometry.

Qualitative and Quantitative NMR. Milligram quantities of MX-analogues (Figure 1 + MBA) were dissolved in deuterated methanol (Aldrich, 99.8 atom %D), and transferred to 5 mm NMR tubes to a height of 60 mm (~1 mL). All spectra were obtained on an Inova 500 MHz NMR instrument. 1,4-Dioxane (Aldrich, 99.8%) was chosen as the internal standard due to its volatility, and ease of removal from the MX analogues after NMR analysis. 1,4-Dioxane interferes with only one chemical shift in MXR. Carbon-13 NMR spectra were obtained for four MX analogues in decoupling mode.

Purity Assay Calculations. Thirty µL of 1,4-Dioxane (density: 1.0337 g/mL) was spiked into 1 mL of deuterated methanol, for a concentration of 30.1 mg/mL in the primary stock solution. Five µL of the primary stock solution was spiked into each NMR sample, which is equivalent to 150.5 µg 1,4-dioxane per sample. The quantitative ^{1}H NMR spectrum of BMX-3 revealed a dioxane peak at δ 3.65 ppm with a peak area equivalent to 8 H's. The peak area of dioxane was then set to 8.00, so that all other areas would be calculated relative to dioxane. The Ring H of BMX-3 at δ 6.35 ppm is equivalent to 1 H with a peak area of 7.03. The weight of BMX-3 in the NMR tube was calculated by Equation 1.

$$W_{unk} = W_{std} \times \frac{N_{std}}{N_{unk}} \times \frac{M_{unk}}{M_{std}} \times \frac{A_{unk}}{A_{std}}$$ (Equation 1, Willard et al., 1988) where

A = peak area
N = number of protons
M = molecular weight
W = weight present.

For BMX-3,

$$W_{BMX\text{-}3} = 150.5\,\mu g \times \frac{8\,H}{1\,H} \times \frac{350.79\,g/mol}{88.11\,g/mol} \times \frac{7.03}{8} = 4.21\ mg\ BMX\text{-}3.$$

The solution in the NMR tube was then transferred to a tared 4 mL amber vial and dried under gentle flow of nitrogen gas. When the deuterated methanol evaporated to dryness, the vial was placed in a vacuum manifold to ensure removal of the solvent. The vial was then weighed on a microscale and the weight of the NMR sample by difference was 5.5 mg. Therefore, BMX-3 is 76% pure as measured by proton NMR. The remaining NMR samples were assessed for purity in the same manner (Table 1). The ox-MX and red-MX standards were prepared without addition of the internal standard dioxane. However, they could still be quantified relative to residual MX remaining in the standard from the synthesis reaction. Ox-MX was found to be 17% pure relative to MX, and red-MX was 88% pure relative to MX, by ^{1}H NMR.

Table 1. Purity of Standards by Quantitative ^{1}H NMR

Compound	Calculated Weight (mg)	Original Weight (mg)	Percent Purity
MX (Sigma)	3.46	5.2	66%
MX ester	1.58	2.64	60%
BMX-1	0.76	4.0	19%
BMX-2	1.06	4.0	27%
BMX-3	4.21	5.5	76%
MCA	4.98	6.0	83%
MBA	6.65	10.4	63%
Ox-MX			17%
Red-MX			88%

The brominated MX-analogues (BMX-1, BMX-2, BMX-3) were synthesized overseas and arrived as one neat 10 mg mixture of BMX-1 and BMX-2, as well as one neat 5 mg BMX-3. Therefore, BMX-1 and BMX-2 had to be separated by high performance liquid chromatographic (HPLC) fractionation (Lloveras et al., 2000). The 10-mg mixture of BMX-1 and BMX-2 was dissolved in 1.5 mL of deuterated methanol (CD_3OD) and the ^{1}H NMR spectrum was obtained by an Inova 500 MHz instrument. The NMR sample was transferred from the NMR tube to a 4 mL amber vial with two successive washes with regular methanol (Burdick & Jackson THM-free methanol). The methanol was evaporated under gentle flow of nitrogen gas. The residue was then diluted to 100 μL and transferred to an HPLC vial with a 350 μL insert. Twenty-five μL aliquots of the BMX mixture were injected onto the Waters HPLC system. The course of the separation was monitored at λ=254 on a photodiode-array detector, using 25:75 acetonitrile (ACN): 0.05 M buffer HCOOH:Et_3N pH 3.2 as the eluent system, at a flow rate of 2.5 mL/min (Beckman Ultrasphere ODS 5 μm x 10 mm x 25 cm). The compounds eluted in the order of, first, an unknown, second, BMX-1, and third, BMX-2. The latter two peak eluates were collected with an automated fraction collector.

Each 35-mL fraction was separately extracted in a 125 mL separatory funnel with two 50 mL aliquots of Ethyl Acetate (Mallinckrodt AR). The aqueous layer was removed

(and stored in the refrigerator in case re-extraction was needed). The organic layer was extracted with 40 mL of brine (DIW saturated with NaCl, Mallinckrodt AR), and the aqueous layer was removed and disposed. The organic layer was dried over a funnel filled with a glass wool plug and ample sodium sulfate (Na_2SO_4, EM Science, Granular), and collected in a round bottom flask. The ~100 mL organic layer was dried down to 1 mL with a rotary evaporator. The remaining 1 mL was loaded onto a preparatory thin-layer-chromatography (TLC) silica plate with a Pasteur pipette and developed for 1 hour with a mobile phase of 1:1 ethyl acetate and hexane (Mallinckrodt AR) in a glass development chamber. BMX-1 gave an R_F value of 0.51, and the R_F of BMX-2 was 0.24.

Compound Identification Confirmation by Direct Probe Mass Spectrometry. The electron ionization mass spectra of MX and red-MX were acquired and confirmed by literature spectra (Kronberg et al., 1991; LaLonde et al., 1990; Padmapriya et al., 1985). The mass spectrum of ox-MX was not previously published, so it is included below (Figure 6). It was found to contain significant contamination from MX (Figure 6, Table 2).

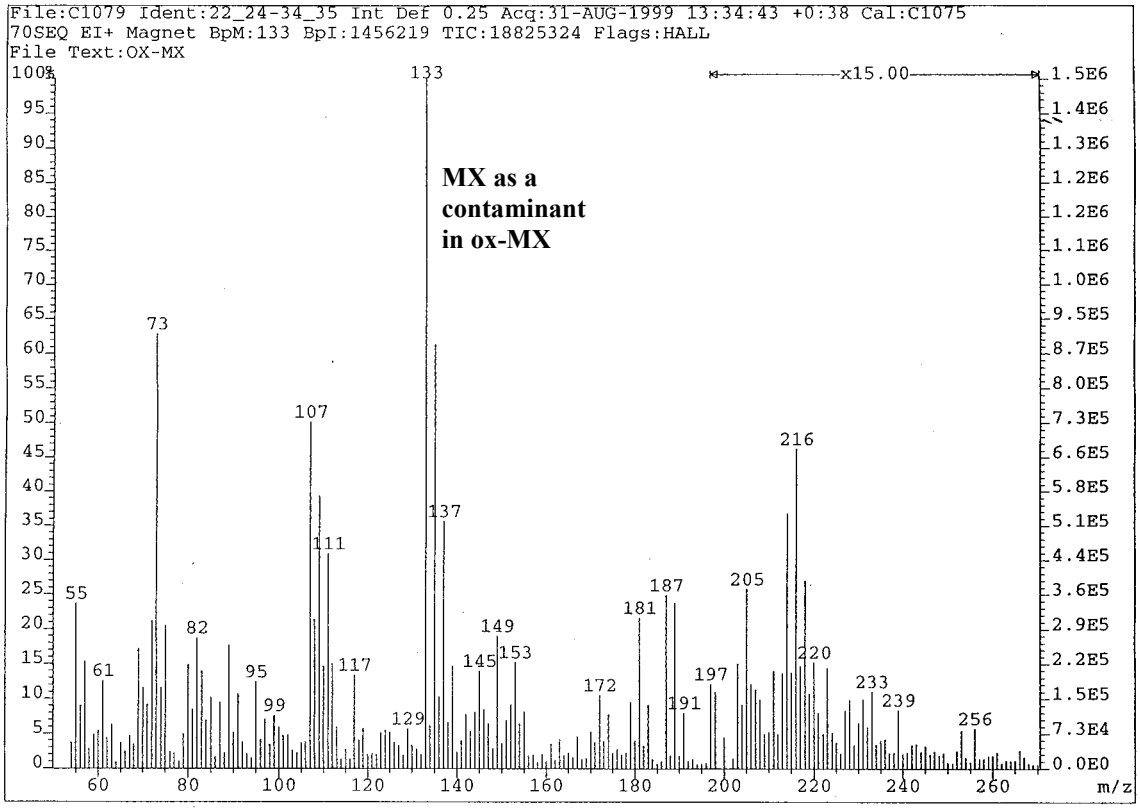

Figure 6. Background-subtracted direct insertion probe EI mass spectrum of synthesized ox-MX (1.81 mg/mL, molecular ion = 232, 17% pure by proton NMR).

Table 2. Ox-MX fragmentation

m/z	Fragment ion
187	$(M\text{-}CO_2H)^+$
133	MX contaminant
107	$C_3HCl_2^+$
73	$C_3H_2Cl^+$

Derivatization of MX-Analogues for GC-ECD and GC/MS Detection. Gas chromatography with electron capture (GC-ECD) and mass spectrometric (GC/MS) detection were chosen as the ideal separation and detection methods for the analysis of MX-analogues because these types of instrumentation are widely used by environmental and water utility laboratories across the United States. However, the majority of the MX-analogues contain one or more hydroxyl groups that can react with unprotected silanol groups on the solid phases of gas chromatographic open tubular columns. Therefore, a methylating agent was chosen to protect the hydroxyl groups of the MX-analogues and allow separation of the MX-analogues on a GC column. The boron-trifluoride methanol complex (BF_3/MeOH, Sigma) was chosen in order to effectively methylate all of the MX-analogues; this is the only methylating agent suitable for ox-MX (Kronberg et al., 1991).

The limiting concentration of BF_3/MeOH was unclear from previous work (Kanniganti et al., 1992), and was evaluated by adding increasing volumes of 14% BF_3/MeOH to a 1 mL solution of MX in methanol (25 μg/L MX/MeOH) (THM-free methanol, Burdick & Jackson). By varying the amount of BF_3/MeOH added, the concentration changed from 7% BF_3/MeOH with a 1 mL addition, to 9% with 2 mL, and 10.5% with 3 mL. Each mixture was sealed with a Teflon-lined, open-top screw cap and heated in a heating block at 70°C (just above the boiling point of methanol, 67°C, to encourage reflux) for 16 hours (Ball, 1998, personal communication). To halt the derivatization reaction after 16 hours, a saturated solution of sodium bicarbonate in deionized water (10% $NaHCO_3$) was added until the pH approached neutral (pH 7). The methylated MX in the neutral solution was then back-extracted with 1 mL of hexane (Ultra-Resi grade 95%, J.T. Baker). The neutral pH of the aqueous fraction ensured that any underivatized MX would remain ionized and dissolved in water, and would not be extracted by hexane. The saturated salt solution (10% $NaHCO_3$), used to neutralize the BF_3/MeOH, has been shown to improve extraction recovery of the esters into hexane (Metcalfe et al., 1966).

When analyzed by GC-ECD on a DB-1701 (30-m x 0.25 mm ID x 0.25 μm film thickness) fused-silica column, the 9% BF_3/MeOH solution gave the largest area response for MXR. Thereafter, a volume ratio of 2:1 BF_3/MeOH to MX/MeOH was utilized for the derivatization step. The final hexane extract was separated on a DB-1701 column with a temperature program of 50°C for 1 min, and 2.5°C/min to 250°C, revealing a retention time of 46.7 min for MXR.

Additional MX-analogues were derivatized with BF_3/MeOH, as outlined above, and analyzed by gas chromatography-ion trap mass spectrometry using both electron ionization (EI) (example in Figure 7, Table 3, ox-MXR) and chemical ionization (CI) modes. The total ion chromatogram and mass spectra obtained for the esterified mucochloric acid revealed two products, MCR *ring* form and MCR *open* form (the methylated 2,3-dichloro-4-oxobutenedioic acid) (Kanniganti et al., 1992; Nawrocki et al., 2000). The two peaks eluted at 12.2 and 20.5 min, on the DB-5 column, with a temperature program of 60°C for 1 min, 2.5°C/min to 250°C, and 250°C for 5 min; injection temperature of 150°C.

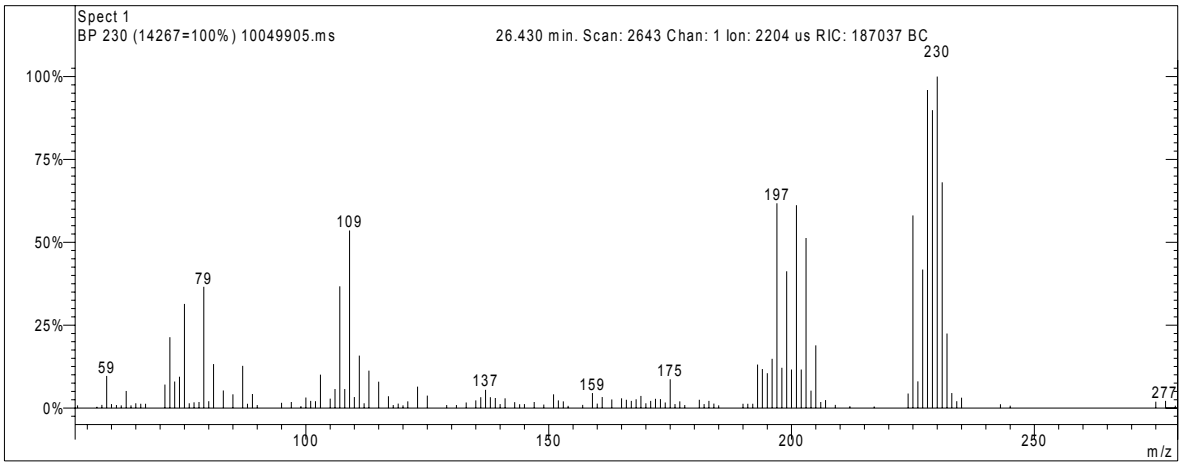

Figure 7. Background-subtracted EI mass spectrum for methylated ox-MX (molecular ion = 260, R_t=26.43 min); agrees with mass spectrum of methylated ox-MX found by Kronberg et al. (1991).

Table 3. Ox-MXR fragmentation

m/z	Fragment ion
229	$(M\text{-}OCH_3)^+$
228	$(M\text{-}CH_3OH)^+$
225	$(M\text{-}Cl)^+$
201	$(M\text{-}CO_2CH_3)^+$
197	$(M\text{-}Cl\text{-}C_2H_4)^+$
109	$C_2H_2O_3Cl^+$
107	$C_3HCl_2^+$
79	CO_2Cl^+

The esterified mucobromic acid also contained two peaks (MBR *ring* and MBR *open* forms) (Backlund et al., 1988; Kronberg et al., 1988; Nawrocki et al., 2000), eluting at 19.17 and 25.73 min. This was also the case for the esterified brominated MX-analogues (BMXR-1 at 25.98 min, BEMXR-1 at 30.70 min, BMXR-2 at 30.14 min, BEMXR-2 at 34.45 min, BMXR-3 at 34.26 min, BEMXR-3 at 37.59 min). The BMX compounds synthesized by Angel Messenguer were not pure. Each one contained three components: an unknown peak, the *ring* form (BMXR) and the *open* form (BEMXR). Identities of these esters were confirmed by spectra in the Ph.D. thesis of Peters (1991).

By GC/MS peak area, red-MX was 66% pure relative to MXR, eluting at 19.08 min, and ox-MXR was 28% pure relative to MXR (Figure 8, Table 4), eluting at 26.43 min. The detector response for red-MX following derivatization was considerably lower due to losses during back-extraction into hexane. Red-MX does not require methylation because it lacks

the hydroxyl group present on the MX ring. The identity of ox-MXR was confirmed by GC/MS (Kanniganti et al., 1992; Kronberg et al., 1991). The mass spectrum of ox-EMXR could not be obtained due to the small amount of available material and detection limit constraints on the Saturn II mass spectrometer. The percent purities of the MXR-analogues are given in Table 5, based on GC/MS peak area.

In order to isolate and quantify EMX, the method required further manipulation. MX was shown previously to isomerize to EMX above pH 4 (Holmbom et al., 1984). Therefore, a pH 6 phosphate-buffered aqueous solution containing MX was monitored over time for production of EMX. Aliquots (1 mL) of this solution were taken at time increments from 10 min to 24 hours, and extracted with methyl *tertiary*-butyl ether (MtBE, OmniSolv grade, EM Science, 1 mL). These MtBE extracts were derivatized with BF_3/MeOH, and extracted with hexane, as outlined above. The hexane extracts were analyzed by GC-ECD and GC/Ion Trap MS on a DB-5 (30-m x 0.25 mm ID x 0.25 μm film thickness, J&W Scientific/Agilent, Folsom, CA) column using a temperature program of 60°C for 1 min, 2.5°C/min to 150°C, and held at 150°C, to encompass the eluting compounds' retention times. Each of the hexane extracts contained three distinct peaks: MXR at 22.85 min, ZMXR at 28.17 min, and EMXR at 29.34 min, as identified by GC/MS (Kronberg et al., 1988). The ratio of MXR to ZMXR to EMXR was 34:15:1, and did not change over the time tested (10 min to 24 hours), as measured by GC-ECD. Therefore, the MX→EMX reaction was not observed at pH 6, unless, of course, the reaction completes in less than 10 min. In subsequent investigations, quantification of EMX was determined against a 2% presence in the MX standard (Table 5). Similarly, quantification of ZMX was determined against a 31% presence in the MX standard.

Derivatization Reaction Time

The optimum derivatization time for MX in the 1-8 hour range was 4 hours with a 65% yield. Aliquots (1 mL) of MX solution (10 μg/mL MX/MeOH) were derivatized with 2 mL of 14%BF_3/MeOH at 70°C for 1, 2, 3, 4, 5, 6, 7, and 8 hours. These results enabled the derivatization time of MX to be reduced from 16 to 4 hours. Then the derivatization time was evaluated for a mixture of other MX-analogues, for 1-8 hours (Onstad and Weinberg, 2001). The mixture contained 250 ng of each MX-analogue dissolved in methanol. Most of the compounds (MX, MCA, MBA, BMX-1, BMX-2, and BMX-3) approached a threshold derivatization efficiency after 3 hours (see Figure 9), with the exception of ox-MX, which will not completely derivatize even after 19 hours. Previous researchers used a derivatization time of 10-16 hours at 70-100ºC in combination with a boron trifluoride methanol complex (Ball, 1998, personal communication; Kanniganti et al., 1992; Kronberg et al., 1991). A derivatization time of 4 hours was chosen for the compounds overall.

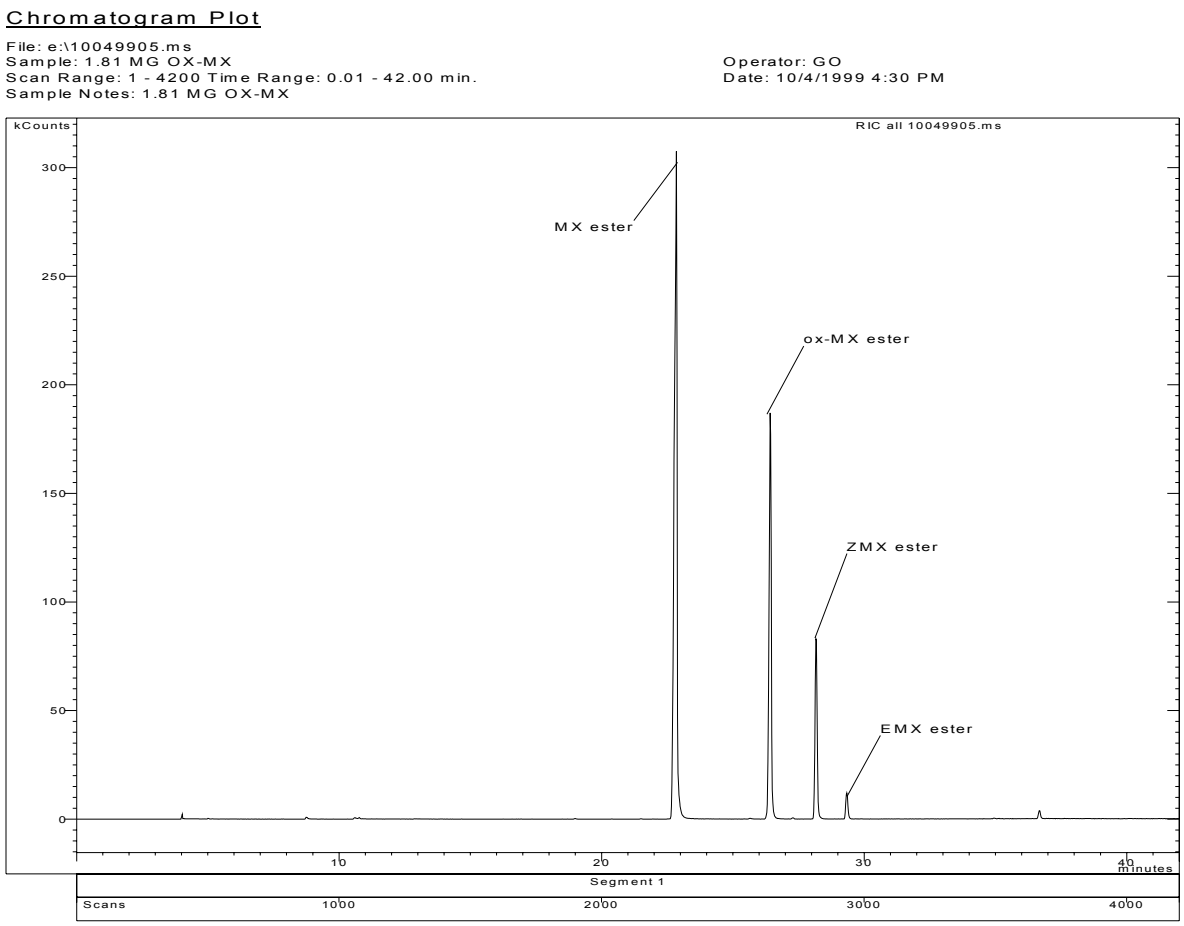

Figure 8. Total ion chromatogram for methylated ox-MX (1.81 mg/mL), with the MX, ZMX and EMX esters in the mixture.

Table 4. Percent Purity of ox-MXR standard

Compound	% TIC	% Area
MXR	52%	58%
Ox-MXR	32%	28%
ZMXR	14%	12%
EMXR	2%	2%

Table 5. Purity of Ester Standards by GC/Ion Trap MS

Compound	Percent purity with respect to components (by area)
MXR	67% MXR, 31% ZMXR, 2% EMXR
Ox-MXR	28% MXR, 58% ox-MXR, 12% ZMXR, 2% EMXR
Red-MX	66% red-MX, 29% MXR, 8% ZMXR
BMXR-1	31% UNK BMX-1, 9% BMXR-1A, 35% BMXR-1B, 25% BEMXR-1
BMXR-2	61% UNK BMX-2, 23% BMXR-2, 16% BEMXR-2
BMXR-3	41% UNK BMX-3, 41% BMXR-3, 18% BEMXR-3
MCR	18% MCR *ring,* 82% MCR *open*
MBR	27% MBR *ring,* 73% MBR *open*

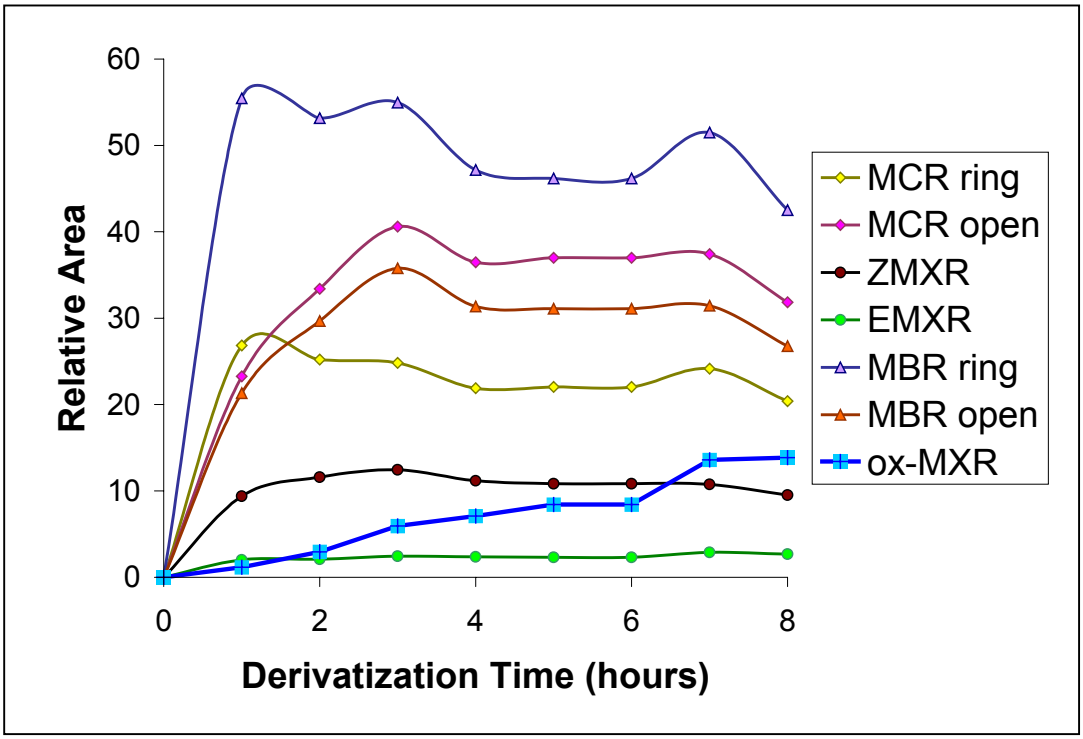

Figure 9. Derivatization of MX-analogues with boron trifluoride/methanol.

Back-Extraction of the MXR-analogues into Hexane

The final step in the analysis was evaluated to determine the recovery of the esterified forms of the MX-analogues during back-extraction from bicarbonate solution to hexane (Onstad and Weinberg, 2001). Synthesized MXR-analogues were dissolved in methanol and spiked into an aqueous sodium bicarbonate solution. Results were attainable for only four of the MX-analogues (Table 6) (red-MX was not extractable by hexane). The equation used to calculate the partition coefficients (K_d) for MXR-analogues between sodium bicarbonate solution and hexane follows (Equation 2):

$$K_d = C_s \Big/ C_a \qquad \text{(Eqn.2)}$$

where K_d = partition coefficient at equilibrium
C_s = concentration of MXR-analogue in hexane (ng/mL)
C_a = concentration of MXR-analogue in sodium bicarbonate solution (ng/mL)

MXR and MCR *open* exhibited the best recoveries by hexane extraction, although only 60% on average (E in Equation 3 and Table 6). Hexane only recovered 7% of the original ox-MXR. Red-MX, when included in this mixture, cannot be recovered at all by hexane. Therefore, other extraction processes are being investigated for red-MX that do not require derivatization prior to GC-ECD analysis. One possibility could be to analyze the MtBE extract directly by GC-ECD, after addition of the internal standard (Kronberg et al., 1991). The fraction of the MXR-analogue extracted (E) was calculated using the following equation:

$$E = C_s V_s \Big/ \left(C_s V_s + C_a V_a\right) \qquad \text{(Eqn. 3)}$$

where E = the fraction of MXR-analogue extracted
V_s = volume of hexane (mL)
V_a = volume of sodium bicarbonate solution (mL)

The "n for 75%" indicates the number of extractions (n) needed to recover 75% of each MXR-analogue. This value is calculated using the following equation (Equation 4), setting E equal to 0.75:

$$n = \frac{\log(1-E)}{\log\left[1 \Big/ \left(1 + K_d V\right)\right]} \qquad \text{(Eqn. 4)}$$

where $V = V_s / V_a$

By adding another hexane extraction and combining the two hexane extracts, MXR and MCR *open* can be more efficiently recovered from the bicarbonate solution. Two hexane extractions are consistent with previous methods for the esterified MX-analogues (Hemming et al., 1986; Kronberg et al., 1991). Recovery of the brominated MXR-analogues is still under investigation.

Table 6. Partitioning of MXR-analogues into Hexane

Compounds	MXR	MCR *open*	ox-MXR	red-MX
Kd	4.75	8.58	0.29	0.00
E (Recovery)	54%	68%	7%	0%
n for 75%	1.77	1.21	19.95	NA

NA: not applicable

Instrument Detection Limits and Gas Chromatographic Separation

A mixture of esterified MX-analogues was separated on a DB-5 column (60-m, 0.25 mm ID, 0.25 µm film thickness) (Figure 10) with a mild temperature gradient (2.5ºC/min) from 105 to 195ºC, followed by a high temperature gradient (20ºC/min) up to 250ºC (Onstad et al., 2000). A shorter column length (30 m) of the same phase did not allow separation between red-MX and the *open* form of mucochloric acid ester (MCR *open*). Coelution was observed between MX and an unknown component in the standard of BMX-2 (BMX-2 UNK). However, this coelution does not preclude detection of MX, because MX can be quantified by the ZMX peak (#14, Table 7), although, with greater variability. Two peaks are present for BMX-1 *ring*, which could be due to the presence of diastereomers, as the ion trap mass spectra appear identical, and the chromatographic retention times are close. Twelve components in the gas chromatogram are listed in Table 7, in addition to red-MX, the three BMX unknowns and the internal and surrogate standards. Use of an HP 6890 GC fitted with a micro electron capture detector (µ-ECD) enabled instrument detection limits of 1 pg/µL for MXR, MCR, ox-MXR, and red-MX; 16 pg/µL for BMXR-1 and BMXR-3; and 25 pg/µL for BMXR-2, in the final hexane extract.

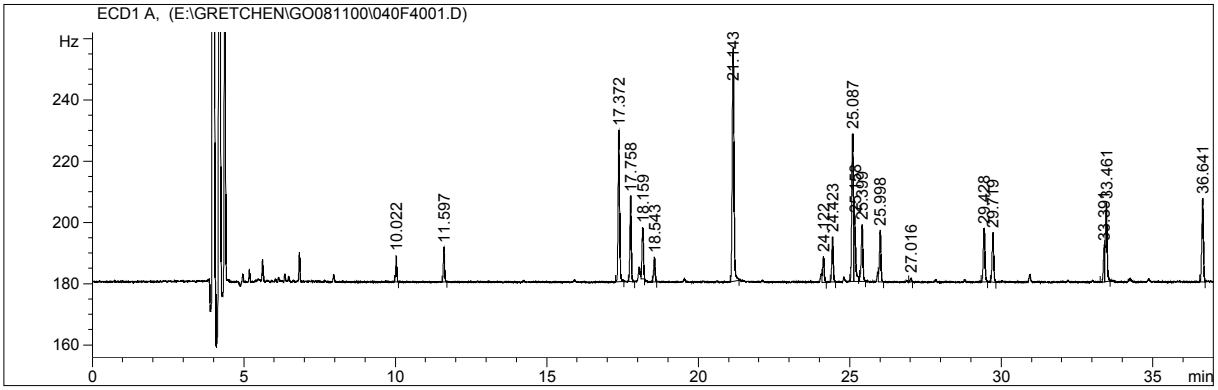

Figure 10. GC-ECD chromatogram of 7 MX-analogues and isomers at 20 pg/µL.

Table 7. Peak identification in GC-ECD trace

Elution Order	Retention Time	Compound
1	10.022	3-Bromochlorobenzene (internal standard, IS)
2	11.597	Mucochloric ester (ring) (MCR *ring*)
3	17.372	unknown component of BMX-1 standard (BMX-1 UNK)
4	17.758	Mucochloric ester (open) (MCR *open*)
5	18.159	Red-MX
6	18.543	Mucobromic ester (ring) (surrogate standard, MBR *ring*)
7	21.143	unknown component of BMX-2 standard (BMX-2 UNK)
8	21.143	MX ester (ring) (MXR)
9	24.122	Ox-MX ester (ox-MXR)
10	24.423	Mucobromic ester (open) (surrogate standard, MBR *open*)
11	25.087	unknown component of BMX-3 standard (BMX-3 UNK)
12	25.158	BMX-1 ester (*ring*) (BMXR-1A)
13	25.399	BMX-1 ester (*ring*) (BMXR-1B)
14	25.998	ZMX ester (ZMXR), an *open* form of MXR
15	27.016	EMX ester (EMXR), an *open* form of MXR
16	29.428	BMX-2 ester (*ring*) (BMXR-2)
17	29.719	BMX-1 ester (*open*) (BEMXR-1)
18	33.391	BMX-2 ester (*open*) (BEMXR-2)
19	33.461	BMX-3 ester (*ring*) (BMXR-3)
20	36.641	BMX-3 ester (*open*) (BEMXR-3)

MX recoveries by other organic solvents, ethyl acetate (EtAc, EM Science, OmniSolv grade) and hexane (Burdick & Jackson, for THM analysis), were compared to MtBE using the 10:2 aqueous solution (100 ng/mL MX/DIW) to organic solvent extraction ratio, and a single extraction. Ethyl acetate (94% recovery) recovered similar amounts of MX as MtBE (83%), while hexane (7%) was relatively unsuccessful at recovering MX from the aqueous solution. The high recoveries of MX (83% MX with MtBE vs. 58% in previous experiment) can be explained by the doubling of the derivatization solvent ratio to LLE extraction solvent (2 mL of 14%BF_3/MeOH to 500 μL of LLE solvent). Thereafter, the LLE extraction solvent was reduced to 500 μL with nitrogen (N_2) gas prior to addition of the derivatization agent. MtBE was chosen as the better extracting solvent over EtAc, because MtBE can be obtained from manufacturers at a higher level of purity; the GC-ECD trace of EtAc contained several contaminant peaks in the vicinity of the MXR elution time.

Liquid-liquid extraction was applied to other MX-analogues, and MtBE was evaluated for recovery of MCA, red-MX, MBA, MX and ox-MX from an aqueous solution (1 ng/mL each in DIW), using the 20:4 extraction ratio, and triplicate extractions. MtBE recovers 40-90% of the MX-analogues (Table 8). This translates to a detection limit of 4-9

pg/µL on column, or 200-450 ng/L in a 20 mL drinking water sample. Red-MX and ox-MX apparently were not recoverable with LLE. ZMX and EMX did not give reproducible area counts for quantitation. Although the LLE recoveries were good for MCR, MBR and MXR, there still existed the need for recovery of the other MX-analogues and preconcentration to achieve lower ng/L levels in drinking water.

Table 8. Percent recoveries of MX-analogues at 1 ng/mL by LLE

Compounds	Percent Recoveries
MCR ring	40%
MCR *open*	57%
red-MX	1%
MBR ring	93%
MXR	81%
ox-MXR	0%
MBR *open*	87%

The MtBE extraction efficiency of MX-analogues from water was next evaluated by comparing recoveries after the addition of salt (granular sodium sulfate, EMScience) or acid [sulfuric acid (Aldrich) to pH 2] (Onstad and Weinberg, 2001). Each extraction was of a 20-mL deionized water sample spiked to 5 µg/L with the MX-analogues. Two standard mixes were evaluated separately, to prevent co-elution on the gas chromatogram, the first one containing MX, ox-MX, and BMX-3, and the second one containing MCA, BMX-1, and BMX-2. Percent recoveries were calculated relative to the GC responses of derivatized standard mixes (Table 9). The MX-analogues were recovered poorly in the control (28 ± 25%), with only three compounds yielding higher that 50% (MXR, ZMXR, and BEMXR-1). The salting-out approach did not improve extraction efficiency relative to the control (16 ± 17%). Acidification to pH 2 improved the MtBE extraction efficiency of both the *open* and *ring* forms of the MX-analogues (74 ± 10%).

Table 9. Extraction Efficiencies of MX-analogues

Compound	Control	Salt	Acid
MCR *ring*	16%	0%	82%
MCR *open*	11%	3%	66%
MXR	61%	39%	89%
ox-MXR	0%	13%	64%
ZMXR	55%	40%	73%
EMXR	12%	14%	61%
BEMXR-1	41%	31%	73%
BEMXR-2	53%	0%	75%
BEMXR-3	0%	0%	87%
average	28%	16%	74%
std dev	25%	17%	10%

Solid Phase Extraction

Solid phase extraction (SPE) was evaluated as a viable method of preconcentration and an alternative method of extraction to LLE. The octadecyl silane phase (C18, J.T. Baker) was compared to LLE for recovery of MX from a 10-mL aqueous solution (100 ng/mL MX/DIW). The aqueous sample was passed through the SPE column at a rate of < 5 mL/min, and the solid phase was dried using a vacuum. When eluted with 1 mL of methanol, the C18 column recovered only 25% of MX in aqueous solution.

Using the method development guidelines of Thurman and Mills (1998), different solid phases and elution solvents were first compared for the recovery of a mixture of MX-analogues made in the elution solvent, and then solid phase recoveries of a mixture of MX-analogues spiked into deionized water and tap water were determined. Two different solid phases, C18 (3 mL, 500 mg) and polyamide (DPA-6S, Supelco, 6 mL, 500 mg) were each washed with MX-analogue solutions (40 ng/mL chlorinated MX-analogues) made separately in methanol (Mallinckrodt AR Anhydrous), MtBE, and 14% BF_3/MeOH (Table 10), to determine whether there would be irreversible retention of the target analytes on the solid phase if these were the eluting solvents used in the SPE process. The BF_3/MeOH esterifying reagent dissolved the polyamide (DPA-6S) phase, and created large air pockets, therefore preventing further investigation of this combination. The BMX compounds were not included in this preliminary study. The percent recovery results follow.

Table 10. Percent recovery of MX-analogues from C_{18} and DPA-6S

Compounds:	**MCR *ring***	**MCR *open***	**red-MX**	**MBR *ring***	**MXR**	**ox-MXR**	**MBR *open***	**ZMXR**	**EMXR**
C18 spk/MtBE	29%	49%	0%	3%	38%	2%	51%	62%	62%
C18 spk/MeOH	108%	59%	0%	102%	122%	70%	56%	78%	148%
C18 spk/BF3/MeOH	60%	53%	0%	63%	65%	0%	29%	48%	70%
DPA-6S spk/MtBE	1%	2%	0%	1%	0%	0%	0%	0%	0%
DPA-6S spk/MeOH	0%	0%	0%	0%	8%	6%	0%	0%	0%

Methanol was chosen to be the best solvent for partitioning of the MX-analogues off of the C18 solid phase extraction columns (average 83% recovery). BF_3/MeOH was the second best solvent for C18 SPE (average 42% recovery), without heating, during derivatization. MtBE gave similar recoveries when applied to C18 SPE (average 36 % recovery). The MX-analogues preferentially partitioned onto the DPA-6S SPE columns using methanol or MtBE (average 0% recovery). The spiked BF_3/MeOH degraded the DPA-6S phase on contact; this is due to the derivatization reaction which releases

hydrofluoric and boric acids. All calculated average percent recoveries were weighted down by zero recovery of red-MX in all cases. For compounds containing *open* and *ring* forms (MXR, MBR, MCR), the *open* forms were retained by the solid phase much more than the *ring* forms (~100% recovery of *ring* vs. ~60% *open* on the C18 spk/MeOH). This was also evident for ox-MXR. The C18 reverse phase proved to be the most effective phase for recovery of the MX-analogues (80-100% recovery of select MX-analogues).

Solutions of MX-analogues in deionized water (100 mL volumes at 1 µg/L MX-analogues/DIW) were then evaluated for recovery by C18 solid phase, with less favorable results. Table 11 highlights the recoveries of MX-analogues under neutral (no alteration, NA) and low pH (acidified to pH 2, AD) conditions, as well as percent breakthrough of columns in tandem (breakthrough from top column was detected in bottom column). Recovery of the MX-analogue standard solution (MeOH Mtx) from C18 solid phase was reevaluated, this time including the BMX compounds. In this case the average percent recovery of the MeOH Mtx was 50-60%, much lower than the above 80-100%. Solid phase extraction was very poor with respect to the BMX compounds, both in the NA and AD solutions. Acidification helped to increase the recovery of the MX-analogues. However, the pH decrease also caused the *ring* forms of the MX-analogues to predominate.

Table 11. Recovery of the MX-analogues from spiked DIW by SPE

Sample label:	**Mtx-NA top**	**Mtx-NA bottom**	**Mtx-AD Top**	**Mtx-AD bottom**	**MeOH Mtx**
Compounds					
MCR *ring*	ND	ND	28%	22%	64%
MCR *open*	ND	ND	ND	ND	53%
red-MX	ND	ND	ND	ND	ND
MBR *ring*	ND	ND	41%	39%	64%
MXR + UNK BMX-2	7%	ND	54%	29%	52%
ox-MXR	ND	ND	26%	ND	46%
MBR *open*	ND	6%	ND	ND	62%
BMXR-1A	ND	>100%	ND	ND	>100%
BMXR-1B	ND	ND	>100%	ND	>100%
ZMXR	ND	ND	ND	ND	54%
EMXR	ND	ND	16%	ND	40%
BMXR-2	>100%	83%	>100%	ND	>100%
BEMXR-1	ND	ND	ND	ND	57%
BEMX-2	ND	ND	6%	ND	65%
BMX-3	ND	ND	ND	ND	ND
BEMX-3	ND	ND	ND	ND	42%

ND: not detected (below 5% recovery), NA: not acidified, AD: acidified to pH 2

A number of other solid phases (3 mL, 500 mg) were then compared to C18 for effective recovery of MX (Table 12). An aqueous solution (260 ng/L MX and 100 ng/L MBA in DIW) was prepared and passed through Cyclohexyl (J.T. Baker), Cyano (J.T. Baker), C8 (Phenomenex Strata), C18E (Phenomenex Strata), and C18 (J.T. Baker) in 250 mL quantities, and results were compared to blanks, both in duplicate. Each column was eluted twice with 500-µL aliquots of methanol. The methanol eluents were derivatized, neutralized, and hexane-extracted before analysis by GC-ECD. None of the solid phases recovered greater amounts of MX than C18 had previously recovered (25%) from spiked DIW. For this reason, SPE was not considered as a practical alternative preconcentration method to LLE for the MX-analogues.

Table 12. Comparison of SPE phases for MX recovery from DIW

Solid Phase	MX Recovery
Cyclohexyl	16%
Cyano	0%
C8	9%
C18E	15%
C18	6%

Method Calibration Curves

The liquid-liquid extraction method was applied to acidified (pH 2), 100 mL samples that were spiked with all of the MX-analogues, except ox-EMX (Figure 1) (Onstad and Weinberg, 2001). The chlorinated tap water samples were quenched of residual chlorine with ammonium sulfate (Mallinckrodt) prior to extraction. The combined 50 mL MtBE extracts (2 x 25 mL MtBE) were reduced to 500 µL with nitrogen gas (UHP, 99.999%). After derivatization of the MtBE extract and neutralization, the final hexane extract (1 mL) recovered only ~60% of the MXR-analogues, considering the results of the partition experiments above. Linearity was observed for MX and MX-analogues in deionized and chlorinated tap waters only at ng/L levels. Example calibration curves are shown in Figures 11 and 12 (MX) and Figures 13 and 14 (MCA). Recoveries of MX and MCA were greatly reduced in the chlorinated tap water samples (Figures 11 and 12), when the detector response was expressed as the ratio of MX or MCA areas to the internal standard (HCB). However, the recoveries were more similar when the detector response was expressed as the ratio of MX or MCA areas to the surrogate standard (MBA) area (Figures 13 and 14). Reliable data is obtainable down to 50 ng/L MCA and 75 ng/L MX by liquid-liquid extraction (100:1 concentration factor) when 100 mL is used as the sample volume.

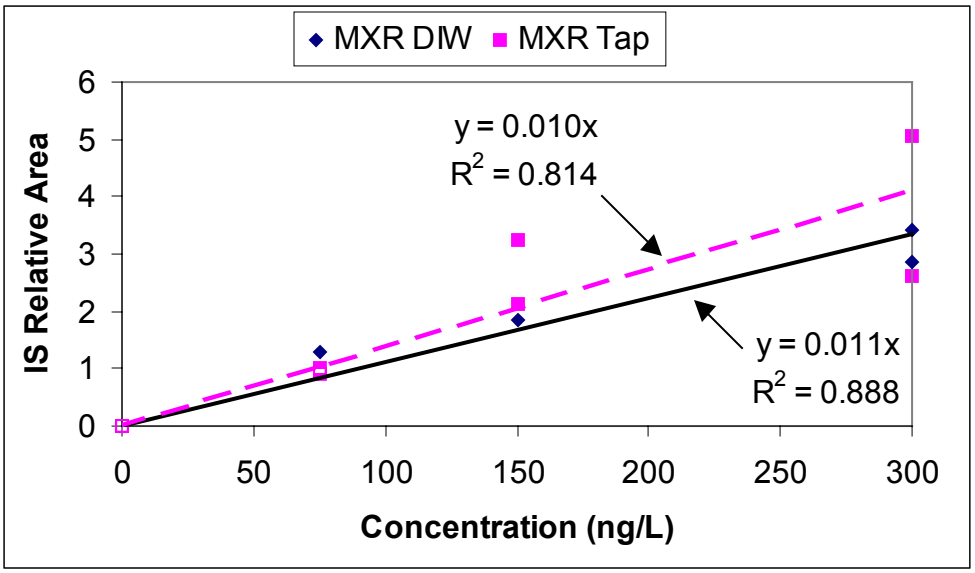

Figure 11. MX Calibration Curve, using area relative to internal standard.

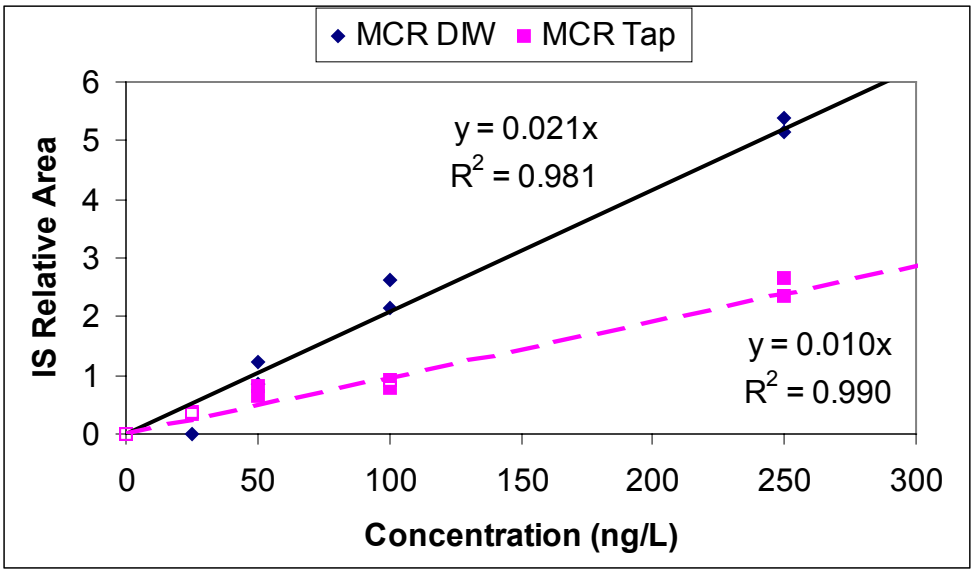

Figure 12. MCA Calibration Curve, using area relative to internal standard.

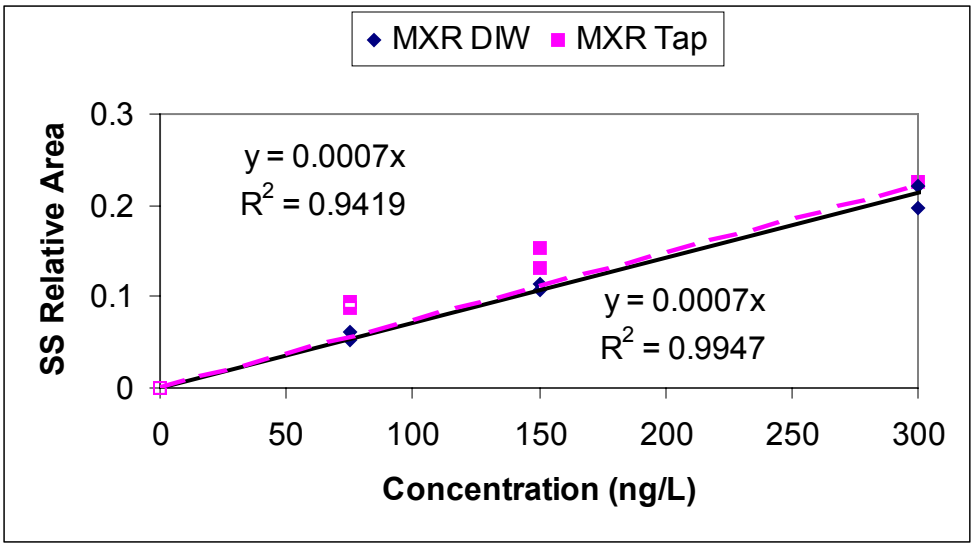

Figure 13. Calibration curve for MX, using area relative to surrogate standard.

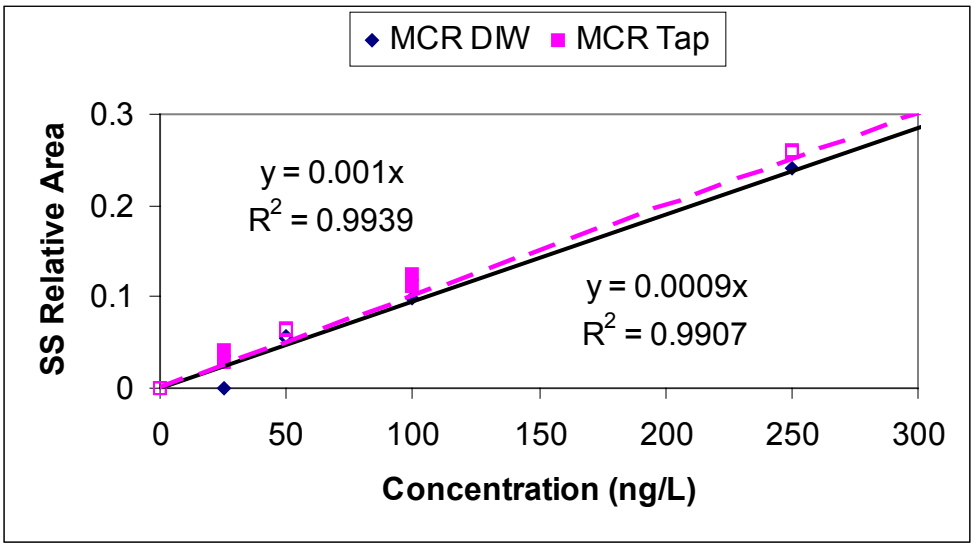

Figure 14. Calibration curve for MCA, using area relative to surrogate standard.

Stability in Aqueous Solutions

In order to stabilize the levels of MX in samples upon collection, they must be quenched of residual chlorine to prevent further production or degradation of MX by chlorine, treated with a biocide to prevent microbial degradation of MX, acidified to pH 2 in order to prevent conversion of MX to *open* forms (ZMX and EMX) and degradation at high pH, and stored at low temperatures (less than or equal to 4°C) to prevent thermal degradation of MX.

Holding temperature of samples was evaluated by storing an aqueous solution (100 ng/mL MX/DIW) at room temperature (25°C) and in a refrigerator (4°C). The samples were extracted after 24 and 48 hours, using LLE at a 10:2 extraction ratio with MtBE. MX was more stable at the lower temperature; at 4°C, 63% MX was recovered, while at 25°C, only 40% MX was recovered. MX recoveries for the two storage temperatures did not change between 24 and 48 hours.

The stability of MX and MCA in tap water samples was then monitored over 14 days to determine the appropriate holding time for samples (Onstad et al., 2000). Previous attempts to determine holding time utilized the biocide sodium azide (NaN_3) in combination with a variety of chlorine quenching agents (ammonium sulfate, *L*-ascorbic acid, sodium sulfite, and sodium bisulfate). However, the MX-analogues could not be recovered by extraction, due to the reaction of sodium azide with the furanone rings in MX-analogues (Beccalli et al., 2000). Therefore, the biocide was removed from the procedure. In this case, a 10 L sample of chlorinated tap water was spiked with MX and MCA to a concentration of 500 ng/L. The water was transferred to 250 mL bottles and quenched of residual chlorine with aqueous ammonium sulfate solution (100 µL of 40 mg/mL $(NH_4)_2SO_4$) or a combination of ammonium sulfate and sulfuric acid.

The samples were stored at 4°C and extracted in duplicate on days 0, 1, 2, 4, 7, and 14. Prior to extraction, each 250-mL sample was spiked with the surrogate standard (MBA) to a concentration of 500 ng/L. The samples containing only ammonium sulfate as the quenching agent needed to be acidified prior to extraction (to pH 3), while the other samples were already acidic (also pH 3). Method calibration samples at concentrations of 0 and 500 ng/L for MX-analogues in deionized water were extracted each day of the study, in order to calculate concentrations of the MX-analogues in the tap water samples. The MtBE extracts were reduced from 100 mL to 500 µL with rotoevaporation and nitrogen gas. After derivatization of the MtBE extract and neutralization, the final combined 2 mL hexane extract (2 x 1 mL hexane) was reduced to 250 µL with nitrogen gas and then spiked with an internal standard, hexachlorobenzene (HCB). This process created a concentration factor of 1000.

The first-order plots show that the combination of ammonium sulfate and acid for quenching stabilized the MX in the tap water samples only slightly longer than ammonium sulfate alone (Figures 15 and 16). The first-order degradation rate constants are very similar, as well ($k \sim 0.077$ days^{-1}, $t_{1/2}=9.0$ days). This agrees with rates of hydrolysis at pH 7.0 measured by Croué and Reckhow (1989) at 20ºC, $k = 0.9 \pm 0.5 \times 10^{-6}$ s^{-1} (~0.07 days^{-1})

and $t_{1/2}$ ~ 8.9 days. The MCA components coeluted with components in the tap water samples and their stability could not be evaluated in this study. The immediate degradation of MX in tap water samples calls for rapid sample extraction and processing upon receipt of samples.

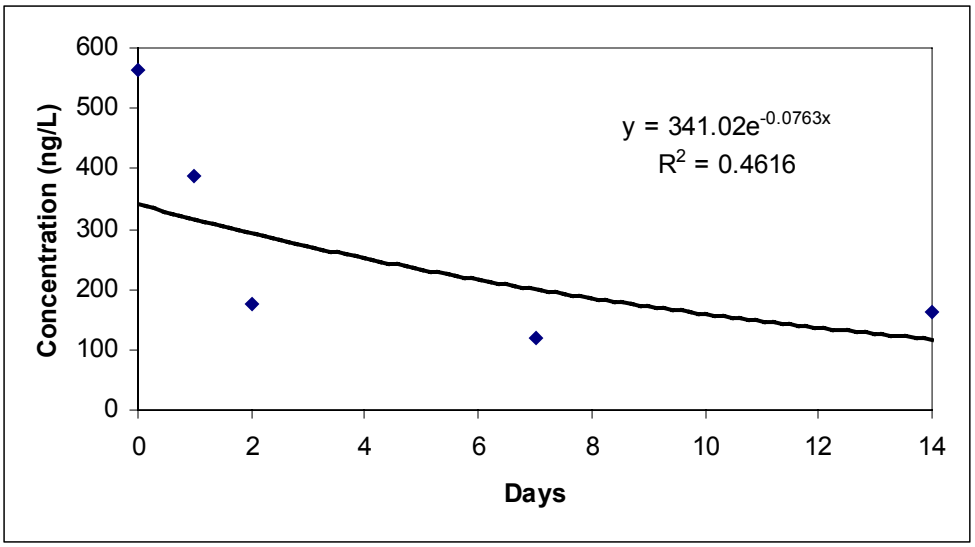

Figure 15. Degradation of MX in chlorinated tap water quenched with ammonium sulfate.

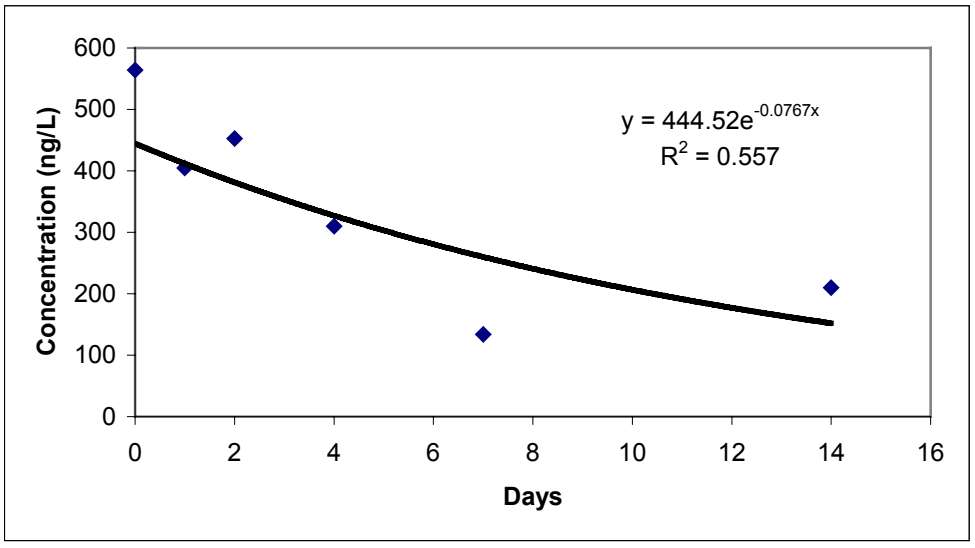

Figure 16. Degradation of MX in chlorinated tap water quenched with ammonium sulfate and preserved with sulfuric acid.

Final Method for Occurrence Study Drinking Water Samples.

The final optimized method developed for the MX analogues is shown in the first part of this chapter (**Method Summary**).

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